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JUL 78 R R COLWELL N00014-75-C-0340
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The biodegradation of naphthalene, creosote, and naphthalene-enriched creosote applied to wood pilings used at Roosevelt Roads Naval Base in Puerto Rico was studied. It was found that naphthalene-degrading bacteria rapidly colonized new wood pilings within hours after installation but that the rate of attachment and subsequent colonization was significantly slower for the naphthalene-enriched creosote-treated wood. The bacteria attaching to new wood were predominantly stalked bacteria whereas the microbial flora of old wood reflected

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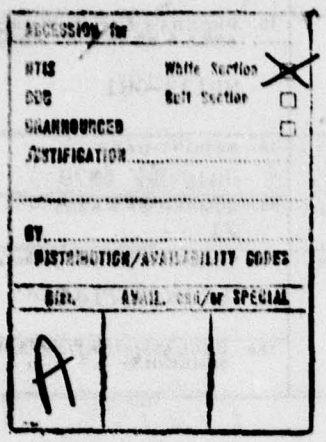
that of the surrounding water and sediment. The gut of Limnoria tripunctata reared in the laboratory was found to be free of bacteria but the external surfaces of the borer were heavily colonized by bacteria.



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MICROBIAL DEGRADATION OF PETROLEUM IN THE MARINE ENVIRONMENT

Contract No. N00014-75-C-0340 P00003

Task No. NR 133-081

Annual Report No. UM/ONR-4

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INTRODUCTION

Aquatic bacteria perform an important role in the natural environment. Bacteria of ecological significance in the marine environment include those that are primary colonizers of substrates, e.g., sand (Meadows & Anderson, 1968), degraders of complex organic compounds, e.g., petroleum (Mulkins-Phillips & Stewart, 1974), naphthalene (Raymond, 1974), and pesticides (Gibson & Brown, 1975), as well as transformers of heavy metals (Nelson & Colwell, 1975), antibiotic resistant (Allen et al., 1977), and contributors to the food chain (Berk et al., 1976). In brief, they are the primary agents of mineralization in the oceans, coastal waters and estuaries.

Biodegradation and biodeterioration of naphthalene and naphthalene creosote-treated wood has been a focus of the research work on this project during the past year. Field work at Roosevelt Roads, Puerto Rico, was carried out to determine the effect of creosote and creosote/naphthalene solutions on the microbial ecology of the water and sediment in the area of pier 3 at Roosevelt Roads. The pilings at Roosevelt Roads Naval Station were replaced in late 1977 with high naphthalene creosote-treated wood. An objective of this research project was to determine the natural flora of water, sediment, and fauna, in particular, the gut flora of the wood borers, before and after the installation of the treated pilings. Environmental effects of naphthalene and naphthalene-treated wood at the microbial level, the ability of the natural microbial communities to biodegrade treated and untreated wood exposed to water and sediment, and the effect of the naphthalene and naphthalene/creosote on the commensal flora of wood-boring organisms were studied. The effects of petroleum, in

enhancing or inhibiting, the biodeterioration of treated and untreated pilings was also considered.

Thus, the specific objectives of the research in the initial phase of the project were achieved. Preliminary work on the biodegradation of naphthalene, creosote, naphthalene-enriched creosote, and petroleum was done. The bacterial flora of beach sand, wood pilings, and Limnoria tripunctata samples collected at Roosevelt Roads was analyzed and bacteria associated with the wooden pilings at the Navy Base were examined to determine the effect of naphthalene-enriched creosote on bacterial attachment and colonization.

MATERIALS AND METHODS

Collection of samples

Water and sediment samples were collected aseptically from two locations within the U.S. Naval Base at Roosevelt Roads, Puerto Rico, which is situated on the western extreme of Puerto Rico in the Caribbean Sea (Fig. 1). One sampling site was within the dockyard at the main pier (Pier 3), which is actively used by ocean going vessels. Salinity of the water at this site was 35 °/oo, at the time of the initial sampling the temperature of the water was 29°C. One side of the pier is constructed from creosote-impregnated wooden pilings. About one-third of the pilings had been installed within three weeks of the sampling, starting in late summer and continuing through the fall of 1977. At the time of the October sampling, construction was in progress. On the other side of the pier, the pilings are in an advanced state of deterioration, resulting from attack by the wood-boring isopod, Limnoria tripunctata. The pilings have been destroyed principally at the air-water interface, with dramatic

destruction of the wood. A control site was selected for the study, located 5 m from the shore line on a deserted beach, approximately 5 km from Pier 3, on the Atlantic side of the island. Water salinity and temperature at the time of the sampling in October were 35 °/oo and 30°C, respectively. Water samples were collected 1 m below the surface using sterile nalgene bottles. Additional samples for chemical analyses were collected in 500 ml aliquots in sterile bottles and filtered through sterile cellulose filters to remove particulate matter. The filtrate was mixed with 100 ml of benzene, shaken for one minute, allowed to settle, and the benzene fraction separated and stored at 4°C. Sediment samples from the control site were collected in nalgene bottles and at the base of the old and new pilings, 3 m from the base of the pilings at Pier 3. Wood samples for microbiological analyzes were also collected. These were cut from the pilings at the air-water interface and at 1 m above and below the air-water interface. Both old and new pilings were sampled. Assistance of Navy SCUBA divers stationed at Roosevelt Roads permitted manual collection of samples at the pier, minimizing disturbance of the water, sediment, and wood surfaces for subsequent microbiological and chemical analyses.

Samples of wood, including 10, 20, 30 and 40% naphthalene-enriched creosote-treated wood, supplied by Dr. David Webb, Koppers Co., were employed in laboratory experiments.

A. Chemical Analyses

Sediment and wood samples were dried at 45°C to remove residual water. Wood samples were pulverized using an analytical micro mill (Model #A-10, Polyscience Corp., Evanston, Ill.). The samples were weighed, placed into a Soxhlet extraction apparatus, and extracted overnight with reagent grade acetone (J. T. Baker).

Following extraction, excess solvent was removed using a Buchi Rotavapor-R rotary evaporator. The extracted material was brought to known volume and analyzed using gas-liquid chromatography.

Benzene extracts of water samples were dessicated using granular $CaSO_4$ (W. A. Hammond Drierite Co., Xenia, Ohio). The dessicant was removed and volume further reduced by rotary evaporation. The extracts were brought to known volume and analyzed using gas-liquid chromatography.

B. Gas-liquid Chromatographic Procedure

All samples were analyzed on a Shimadzu GC-4BMPF gas-liquid chromatograph (Shimadzu Seisakusho Ltd., Kyoto, Japan), fitted with a flame ionization detector. A 12 ft column packed with 3% SE-30 adsorbed onto 60/80 Chromosorb WAW-DMCS (Supelco Inc., Bellefonte, Pa.) was used throughout the analysis. Operating parameters of the GLC analysis were as follows: carrier gas N_2, 60 ml/min; injector temperature, 300°C; detector temperature, 300°C; and temperature profile, 70°C → 270°C @ 2°C/min.

Peak area was computed using a Hewlett-Packard 3373B integrator (Hewlett-Packard, Avondale, Pa.).

Co-chromatography was used to identify the naphthalene peak, with an average error of ± 10 sec between consecutive analyses.

C. Bacteriological Analyses

Samples for bacteriological analyses were examined within 15 min of retrieval. Other samples were stored at 4°C. Serial dilutions of sediment, water, and wood were prepared to 10^{-5}, 10^{-3}, and 10^{-3}, respectively, using 9 ml aliquots of marine salts solution (2.4% (w/v) NaCl, 0.7% (w/v) $MgSO_4 \cdot 7H_2O$, 0.075% (w/v) KCl). Aliquots (0.1 ml) of the diluted samples were pipetted onto plates of 2216 agar (Difco) and half-strength 2216 agar (laboratory prepared 2216 agar, containing half the quantities of peptone

and yeast extract of standard commercially prepared 2216 agar). This medium was used because of evidence from our laboratory showing that lower nutrient levels in media enhances growth of many bacterial spp. otherwise not recovered from aquatic samples (Mallory et al., 1977). Three replicates of each dilution were plated and the inoculated plates were incubated at ca. 27°C for 5 days. Plates containing between 30 and 300 colonies were used to randomly select colonies for streaking onto plates of 2216 agar, with three subsequent serial streakings of each culture being done to ensure purity of the strains.

Heat-fixed smears of the pure cultures were stained using Hucker's modification of the Gram stain (Hucker & Conn, 1923). After purification, the cultures were inoculated onto 2216 agar slopes. Any culture suspected to be mixed was successively re-streaked until pure cultures were recovered, as determined by examination of colonial morphology under phase contrast microscopy. Original isolates were maintained in stock culture on 2216 agar slopes at 4°C. Subcultures of the working bench cultures were prepared every 12 weeks. During subsequent testing, viability of three cultures was lost. The 322 isolates carried through the full testing series are listed in Table 1. Sixteen reference cultures included in this study were maintained as described above for the marine isolates.

Each strain was examined for 107 biochemical, cultural, morphological, nutritional and physiological characters. Media were inoculated from 48 hr cultures grown on 2216 agar and were incubated at 25°C for 7 days. Whenever possible, 2216 agar was used as the basal medium for the test media. Otherwise, media were prepared in marine salts solution. To assess possible test error, 10 strains were examined in duplicate, otherwise, tests were carried out once and were repeated only in the event of

inconclusive results. The tests used in the analysis have been described previously (Colwell & Weibe, 1970; Austin et al., 1977).

D. Coding of Data

The characters were coded '1' for positive or present, '0' for negative or absent, and '9' for non-compatible or missing. The final n x t matrix contained 337 strains and 107 characters.

E. Computer Analyses

The data were analyzed using the simple matching coefficient (S_{SM}; Sokal & Michener, 1958) which includes both positive and negative matches, and the Jaccard coefficient (S_J; Sneath, 1957) which excludes negative matches. Clustering was by unweighted average linkage (Sneath & Sokal, 1973). The hypothetical median organism for each cluster was also calculated (Liston et al., 1963). Programs employed included TAXAN6, UMDTAXON3 and IGPS3 program packages available at the University of Maryland UNIVAC 1108 computer.

F. Identification of the Phenetic Groups

Phena, not containing reference cultures, were identified as far as possible using the diagnostic keys in Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons, 1974) and tables of Cowan (1974).

Electron microscopy

1) Discs of untreated wood, and wood impregnated with 10% and 30% naphthalene-containing creosote were mounted on aluminum scanning electron microscope (SEM) stubs and these were suspended on a frame 1m below the water's surface at the Pier 3 sampling site (Zachary, Taylor, Scott & Colwell, in press). Samples of each wood type were removed from the water after exposure to the water for 2 and 4 days. The samples were

immediately fixed in a 2% (w/v) buffered gluteraldehyde solution (pH 7.4), and subsequently prepared for SEM observation, including ethanol dehydration, critical point drying under carbon dioxide, and sputter coating with gold-palladium alloy.

2) Electron microscopy of microorganisms associated with Limnoria tripunctata

Limnoria tripunctata were maintained in aquaria containing artificial seawater (Instant Ocean) and blocks of white pine wood. Specimens of the macroorganisms were collected from Limnoria-infected wood and placed in 2% buffered gluteraldehyde prepared with artificial seawater.

Whole specimens, after fixation for 18-24 hours (4°C), were prepared for SEM examination, following the procedures described above. To obtain gut samples, isopods taken from the aquaria were placed in buffered gluteraldehyde and the digestive tracts were dissected and transferred to vials containing fresh fixative. After fixation in gluteraldehyde for 1 hour at 4°C, the digestive tracts were fixed for an additional 18-24 hours at 4°C, in 0.1 M cacodylate buffered (pH 7.4) 1% (w/v) osmium tetraoxide. Gut samples for SEM were treated as described above. However, prior to metal shadowing, the critical point dried gut specimens were affixed to a SEM stub and cut open with a razor blade.

For transmission electron microscopy (TEM), fixed isopod gut samples were dehydrated, using a graded acetone series and embedded, in known orientation, in Epon 812 epoxy plastic. Thin sections cut through the plastic-embedded digestive tracts were stained with uranyl acetate and lead citrate and examined using a Hitachi HU 11-A electron microscope.

The same procedures were used to prepare whole specimens and gut samples of Limnoria, from animals collected in situ at Roosevelt Roads,

except that the initial fixation period in buffered gluteraldehyde was 5-7 days at 25 to 27°C.

RESULTS

Chemical analyses

1) Water

Analysis using gas-liquid chromatography revealed no detectable traces of naphthalene in either water or sediment samples collected at the control station or at Pier 3. High molecular weight compounds were evident at both stations, most probably materials arising from decay of organic matter, e.g., humic acid and fulvic acid.

2) Sediment

Preliminary results indicate that only traces of naphthalene are present in the sediment at Pier 3.

3) Wood piling

Rsults of analyses of the wood piling samples are given in Table 2. No detectable naphthalene could be found in wood cut from the top of the old pilings and only trace levels were observed in the top wood from new pilings. A steady increase in naphthalene content was apparent in both new and old wood, moving from the top of the piling to the intertidal zone and to the bottom of the piling at the water-sediment interface.

Taxonomy of the marine bacterial flora of the water, sediment and wood

A. Clustering of the Strains

Results obtained using the S_J coefficient were confirmed using the S_{SM} coefficient and indicated that 330 strains, which was 93% of the total, and the reference strains, including Bacillus cereus, B. megaterium and

B. subtilis, could be recovered in 31 clusters, each defined at or above the 85% similarity (S) level. Most strains examined in this study, that is, 206 strains, were recovered in seven of the major clusters, which could be identified as B. megaterium, Flavobacterium sp., Hyphomicrobium sp., Pseudomonas sp., Vibrio sp. and unidentified sheathed bacteria. The remaining clusters each contained less than 9 strains, and were identified as Bacillus, Caulobacter, Hyphomonas, Pseudomonas and Vibrio spp.

B. General Characteristics of the Strains

The strains were well suited for the marine environment at Puerto Rico, insofar as they grew best at higher temperatures, i.e., 37°C (97%) and 42°C (94), but not at 4°C (0%). All strains grew in the presence of 2% (w/v) sodium chloride, and most tolerated 5% sodium chloride (w/v) (88%) and 7.5% sodium chloride (w/v) (69%), with a few producing colonies in 15% sodium chloride (w/v) (14%). The majority of the isolates were Gram-negative (59%); some demonstrated very tapered ends or stalks (34%) upon initial isolation, and were motile (78%), proteolytic (67%) rods, producing catalase (96%) and oxidase (99%), readily hydrolyzing casein (74%), Tween 20 (78%) and Tween 40 (89%), but not degrading cellulose (8%), chitin (1%) or urea (1%).

Of the compounds tested, only the amino acids, including alanine (59%) and proline (64%), were readily utilized; whereas carbohydrates, particularly lactose (13%), melezitose (21%) and xylose (12%) were not. A positive result for carbohydrates was often borderline growth or no growth, when compared with carbon-free controls which showed no growth at all.

It was observed that strains derived from a given sample, e.g., in water from the unpolluted beach, gave collective responses to certain

characters. For example, all strains recovered from water at the control site, were Gram-positive, fermentative rods. Of particular interest, however, were the distinctions between the characteristics of bacteria isolated from new and old wood. As a group, organisms from new wood were all Gram-negative, stalked rods which reduced nitrite (92%) and grew in the presence of 10% (w/v) sodium chloride (96%), and were sensitive to penicillin, but did not degrade blood, casein, gelatin, starch or tyrosine, or utilize serine. In contrast, the strains from old wood, apart from being Gram-negative rods, were more reactive. They were haemolytic (75%), proteolytic (92%), degraded casein (96%), starch (76%) and tyrosine (92%), reduced nitrate to nitrite (72%) but no further, utilized serine (84%) and were resistant to penicillin (77%) but did not grow in 10% (w/v) sodium chloride. Such differences were not apparent amongst the other ecological groupings of strains.

C. Distribution of the Organisms in the Marine Environment

The greatest diversity of bacterial taxa, a total of 28, was isolated from the sediment samples, from which 20 phena were recovered exclusively from sediment. Phena 13 (Bacillus megaterium) and 17 (unidentified sheathed organisms) were the most widely distributed amongst the samples taken in this study. In contrast, 13 phena were specific to a given sample (Table 3).

From the new wooden pilings, Hyphomicrobium neptunium (phenon 22) was recovered in almost pure culture and just under half of the strains (45%) utilized naphthalene. These organisms were not found in the water column or on the old, deteriorated pilings. The old pilings did not possess an unique microflora but possessed an associated flora reflective of that of the water and sediment (Table 2).

Electron microscopy

The short exposure periods, i.e., 2 to 4 days, were sufficient to allow microbial colonization of all the test surfaces, indicating that microfouling is an extremely rapid process in the tropical marine environment at Puerto Rico. Although all surfaces were colonized, the rapidity and extent of microfouling was greater on the untreated wood than on the naphthalene/creosote-treated wood, but no differences in microfouling were evident between 10 and 30% naphthalene/creosote-treated wood. After 2 days in the water, the untreated wood surface was covered by extensive colonies of floc-forming bacteria, which appeared as mat-like coverings at low magnification, but were clearly visible as bacteria enmeshed in an extensive fibrillar network, when viewed at higher magnification. In contrast, on the naphthalene/creosote-treated wood, at 2 days, bacteria demonstrating only a few fibrils were observed and extensive mat-like colonies were not yet visible. After 4 days in the water, however, large mat-like colonies of floc-forming bacteria were evident on the naphthalene/creosote-treated wood. Thus, microbial succession appeared to be similar on treated and untreated wood, but colonization occurred more slowly on the naphthalene/creosote-treated surfaces.

The more rapid microfouling of untreated wood was clearly evident from heavy bacterial growth and presence of large numbers of diatoms observed, after exposure of the wood 4 days in the water. Diatoms were not observed to occur within this time period on the naphthalene/creosote-treated wood. It is possible that naphthalene/creosote treatment has a specific inhibitory or toxic effect on eucaryotic diatom cells, but not on procaryotic bacterial cells. Future studies will be aimed at resolving this question.

One striking result of these studies is that floc-forming bacteria were found to play a dominant role in the initiation of biofouling of wood surfaces at Roosevelt Roads. Future experiments, using a variety of different materials, will be aimed at determining whether these floc-forming bacteria are dominant colonizers only on wood surfaces or represent the dominant periphytic microorganisms attaching to all types of submerged surfaces.

SEM analysis revealed that the exoskeleton of L. tripunctata maintained in laboratory aquaria or obtained from pilings in the field is subject to heavy bacterial colonization, especially in the area of the pleotelson. The digestive tracts of these isopods were filled with pieces of wood. However, in spite of the presence of a dense external bacterial flora, the gut of Limnoria reared in the laboratory was found to be free of bacteria or other microorganisms. Examination of Limnoria specimens collected at Roosevelt Roads revealed that there was no microflora associated with the gut lining, as has been observed for termites (Bloodgood & Fitzharris, 1976; Breznak & Pankratz, 1977). However, bacteria were observed in the gut contents when the gut was examined by thin section transmission electron microscopy. Future studies of Limnoria Populations occurring in situ will be carried out to continue this observation. Also, studies will be aimed at determining whether bacteria seen in the gut contents of the Limnoria serve as food for the Limnoria and/or play a symbiotic role in the wood degrading process.

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Table 1. Source of bacterial strains included in the study[a]

Station	Sediment	Water	Wooden pilings		No. of strains studied
			Old	New	
Control (unpolluted)	52	13	--	--	65
Pier (dockyard)	--	25	25	25	
at base of old pilings	49	--	--	--	
3 m from old pilings	39	--	--	--	257
at base of new pilings	48	--	--	--	
3 m from new pilings	46	--	--	--	
Total number of strains	234	38	25	25	322

[a]Number of strains selected from dilution plates after incubation of the plates at ca. 27°C for 5 days.

Table 2. Naphthalene content of creosote pilings

Piling sample	Sample location	Naphthalene content (mg/gm piling)
New	0 meter	0.63*
	1 meter	12.75 ± 0.11
	3 meters	21.30 ± 0.57
Old	0 meter	N.D.
	1 meter	5.22 ± 0.25
	3 meters	7.79 ± 0.32

*Only one sample analysis revealed naphthalene at a significant concentration.

N.D. = Not detectable.

Table 3. Recovery of phenetic groups (%) from the marine samples

Phenon	Total number of strains[a]	Isolated from[b]								
		NPS_0	NPS_3	OPS_0	OPS_3	PW	NW	OW	CS	CW
1	4	50	0	0	50	0	0	0	0	0
2	5	0	0	0	0	10	0	85	0	0
3	3	0	0	0	0	100	0	0	0	0
4	11	36	9	0	0	0	18	36	0	0
5	8	12	0	0	0	75	0	0	12	0
6	2	0	50	0	0	0	0	0	50	0
7	9	0	0	11	0	0	0	0	89	0
8	8	0	0	0	0	0	0	0	100	0
9	2	0	100	0	0	0	0	0	0	0
10	3	0	0	0	0	0	0	0	100	0
11	2	0	0	0	0	0	0	0	100	0
12	6	50	0	50	0	0	0	0	0	0
13	85	14	25	13	21	15	0	0	2	9
14	2	0	0	0	0	0	0	0	0	100
15	7	0	0	29	0	0	0	0	71	0
16	7	100	0	0	0	0	0	0	0	0
17	49	18	8	39	20	0	2	4	8	0
18	3	0	100	0	0	0	0	0	0	0
19	2	100	0	0	0	0	0	0	0	0
20A	3	33	0	33	33	0	0	0	0	0
20B	4	0	0	25	0	0	0	25	50	0
21	3	0	33	0	33	0	0	0	33	0
22	22	0	0	0	0	0	100	0	0	0

Table 3 (continued)

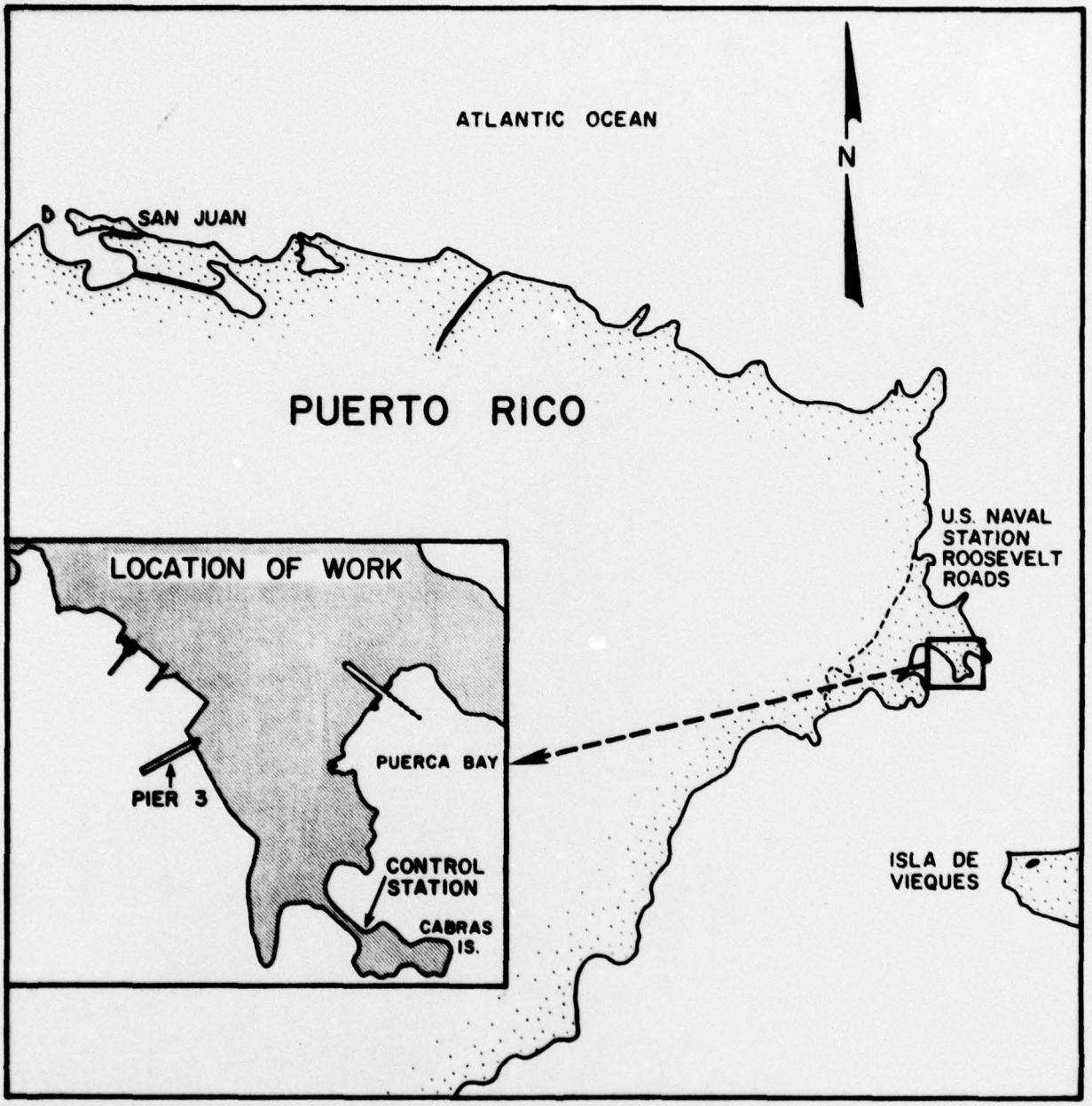
Phenon	Total number of strains[a]	Isolated from[b]								
		NPS_0	NPS_3	OPS_0	OPS_3	PW	NW	OW	CS	CW
23	4	25	75	0	0	0	0	0	0	0
24	3	0	0	100	0	0	0	0	0	0
25	3	0	0	100	0	0	0	0	0	0
26	3	0	67	0	33	0	0	0	0	0
27	2	0	0	0	50	0	0	0	0	50
28	2	0	0	50	0	0	0	0	50	0
29	6	0	0	0	33	0	0	0	50	17
30	10	0	0	0	10	0	0	0	80	10
31	2	100	0	0	0	0	0	0	0	0

[a] Excluding reference cultures.

[b] NPS_0, NPS_3, OPS_0, OPS_3, PW, NW, CW, CS, and CW represent new piling sediment at 0 m and 3 m, old piling sediment at 0 m and 3 m, pier water, new piling wood, old piling wood, control sediment and control water, respectively.

FIGURE LEGEND

Fig. 1. Sampling sites of the microbial ecology studies


ATLANTIC OCEAN
N
SAN JUAN
PUERTO RICO
U.S. NAVAL STATION ROOSEVELT ROADS
LOCATION OF WORK
PUERCA BAY
PIER 3
CONTROL STATION
CABRAS IS.
ISLA DE VIEQUES